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Xenobiotic Biotransformation in Unicellular Green Algae[1]

Involvement of Cytochrome P450 in the Activation and Selectivity of the Pyridazinone Pro-Herbicide Metflurazon

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The *N*-demethylation of the pyridazinone pro-herbicide metflurazon into norflurazon implies a toxification in photosynthetic organisms. This is confirmed by quantitative structure activity relationships determined for two unicellular green algae, *Chlorella sorokiniana* and *Chlorella fusca*; however, the latter is 25 to 80 times more sensitive to metflurazon. This sensitivity is linked to differences in the *N*-demethylase activity of both algae, as determined by an optimized in vivo biotransformation assay. Apparent K_m values of the metflurazon-*N*-demethylase indicate a 10-fold higher affinity for this xenobiotic substrate for *Chlorella fusca*. Furthermore, algal metflurazon-*N*-demethylation is characterized by distinct variations in activity, depending on the stage of cell development within the cell cycle. Several well-established inhibitors of cytochrome P450-mediated reactions, including piperonyl-butoxide, 1-aminobenzotriazole, 1-phenoxy-3-(1H-1,2,4-triol-1yl)-4-hydroxy-5,5-dimethylhexane, and tetcyclacis, as well as cinnamic acid, a potential endogenous substrate, inhibited the *N*-demethylation of metflurazon. The results suggest that the *N*-demethylation of metflurazon by both algae is mediated by a cytochrome P450 monooxygenase. The determination of antigenic cross-reactivity of algal proteins with heterologous polyclonal antibodies originally raised against plant P450s, anti-cinnamic acid 4-hydroxylase (CYP73A1), anti-ethoxycoumarin-*O*-dealkylase, anti-tulip allene oxidase (CYP74), and an avocado P450 (CYP71A1) or those of bacterial origin, CYP105A1 and CYP105B1, suggests the presence of distinct P450 isoforms in both algae.

In addition to their involvement in distinct biosynthetic pathways, plant Cyt P450 enzymes play a central role in the response to foreign compounds, including a variety of herbicidal agents (for reviews, see Durst and Benveniste, 1993; Bolwell et al., 1994). P450-mediated chemical alteration of a herbicide usually results in reduced biological activity. Such phase-I biotransformations are one of the most important mechanisms in biological detoxification. There are, however, examples of increased phytotoxicity following phase-I biotransformation steps that are presumably associated with P450 activity (reviewed by Cole, 1994). The ability to (de)toxify a herbicide is one basis for herbicide selectivity and differential sensitivity between plant species.

In the unicellular green alga *Chlorella fusca*, the pyridazinone pro-herbicide metflurazon requires *N*-demethylation to form the active derivative norflurazon, a potent inhibitor of phytoene desaturase involved in carotenoid biosynthesis (Tantawy et al., 1984; Fig. 1). On the other hand, a second *N*-demethylation step, forming the demethyl derivative SAN 9774, implies an efficient detoxification. The involvement of plant P450s in the *N*-demethylation of herbicides has been documented in only a few cases (Frear et al., 1969; Fonné-Pfister et al., 1988; Mougin et al., 1990) but not yet with regard to metflurazon.

In previous papers, the involvement of P450s in the biotransformation of foreign compounds by algae was demonstrated using alkoxycoumarins and alkoxyresorufins as model substrates (Thies and Grimme, 1994, 1995). The variability in the respective *O*-dealkylase activities between the two algal strains we examined, *C. fusca* and *Chlorella sorokiniana*, attracted interest in the question of whether similar properties in *N*-demethylation might be a determinant of herbicide selectivity in the case of metflurazon.

In this report we describe differential sensitivities of *C. fusca* and *C. sorokiniana* by quantitative structure-activity relationships determined for a congeneric group of pyridazinone herbicides, metflurazon, norflurazon, and SAN 9774. By using an optimized in vivo biotransformation

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Abbreviations: ABT, 1-aminobenzotriazole; AOS, allene oxide synthase; ARP-1, purified avocado P450 polypeptide; BAS 111..W, 1-phenoxy-3-(1H-1,2,4-triazol-1yl)-4-hydroxy-5,5-dimethylhexane; CA4H, cinnamic acid 4-hydroxylase; EC_{50}, effector concentration for 50% response; ECOD, ethoxycoumarin-*O*-dealkylase; 7-ER, 7-ethoxyresorufin; EROD, ethoxyresorufin-*O*-dealkylase; K_m(app.), apparent Michaelis constant; metflurazon (SAN 6706), 4-chloro-5-dimethylamino-2-(α,α,α-trifluoro-*m*-tolyl)pyridazin-3(2H)-one; norflurazon (SAN 9789), 4-chloro-5-methylamino-2-($\alpha\alpha\alpha$-trifluoro-*m*-tolyl)pyridazin-3(2H)-one; P450, Cyt P450 enzyme; PBO (piperonylbutoxide), 2-(2-butoxyethoxy)ethyl-6-propylpiperonyl ether; SAN 9774, 4-chloro-5-amino-2-($\alpha\alpha\alpha$-trifluoro-*m*-tolyl)pyridazin-3(2H)-one; SU1 (SU2), sulfonylurea *N*-dealkylating P450s from *S. griseolus*; t_x, sampling time (t_0, t_1, etc.); tetcyclacis, 5-(4-chlorophenyl)-3,4,5,9,10-penta-azatetracyclo-[5.4.$10^{2,6}$, $O^{8,11}$]dodeca-3,9-diene.

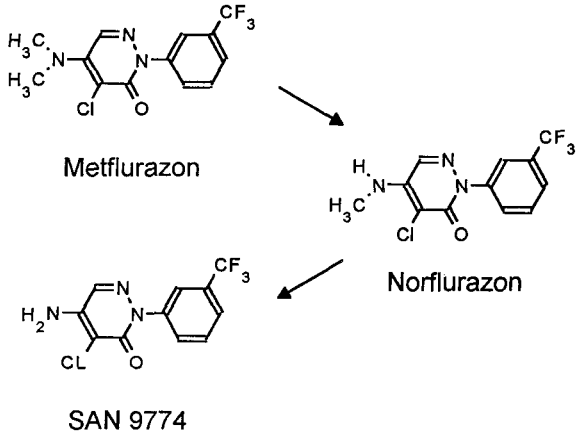

Figure 1. Successive *N*-demethylation of the pyridazinone herbicide metflurazon in *C. fusca*.

assay, we demonstrate that metflurazon selectivity is linked to distinct kinetic properties with respect to the *N*-demethylation step. The inhibition of *N*-demethylation effected by several P450 inhibitors (piperonylbutoxide, ABT, BAS 111..W, and tetcyclacis) indicates that the *N*-demethylation of metflurazon, determined for both algae, is a P450-dependent reaction.

Antigenic cross-reactivity of algal proteins with heterologous polyclonal antibodies originally raised against plant or bacterial P450s might give evidence of the presence of distinct P450 isoforms in both algae. The antibodies tested included anti-CA4H (CYP73A1), originally reported by Werck-Reichhart et al. (1993), and an anti-ECOD (Batard et al., 1995), both from *Helianthus tuberosum*; anti-AOS (CYP74) from *Tulipa gesneriana* (Lau et al., 1993), and anti-ARP1 (CYP71A1) from *Persea americana* (O'Keefe and Leto, 1989). Anti-SU1 (CYP105A1) and anti-SU2 (CYP105B1) are from bacterial antigens of *Streptomyces griseolus* (O'Keefe et al., 1988; Omer et al., 1990). It is noteworthy that, except for AOS, P450s originally detected by these antibodies have already been documented to be participants in the biotransformation of distinct xenobiotics (O'Keefe et al., 1988; O'Keefe and Leto, 1989; Pierrel et al., 1994).

MATERIALS AND METHODS

Chemicals

The pyridazinones metflurazon, norflurazon, and SAN 9774 were a gift from Sandoz (Basel, Switzerland). 7-ER was synthesized from resorufin by the method of Prough et al. (1978) as modified by Klotz et al. (1984) and was judged to be greater than 98% pure by HPLC using a combination of diode array and fluorescence detection. Sources of other chemicals were as follows: ABT, Aldrich; BAS 111..W and tetcyclacis, BASF (Limburgerhof, Germany); cinnamic acid, Fluka; 4-(2-aminoethyl)-benzolsulfonylfluoride, hydrochloride, Boehringer Mannheim; piperonyl butoxide and organic solvents (analytical reagent or HPLC grade), Riedel-deHaen (Seelze/Hannover, Germany). Ingredients required for algal nutrient media were obtained from Merck (Darmstadt, Germany).

Organisms and Culture Conditions

The unicellular green alga *Chlorella fusca* var *vacuolata* Shih. et Krauss, strain 211–15, culture collection Pringsheim (Göttingen, Germany), was grown photoautotrophically at 28 ± 0.5°C in an appropriate sterilized medium (Grimme and Boardman, 1972) adjusted to pH 6.4 (10 mM sodium phosphate buffer). Cultures were aerated with sterilized, water-saturated air, enriched with CO_2 (1.5–2.0%, v/v), and illuminated by a combination of two types of fluorescent tube lights (L36W/41 Interna, L36W/11 daylight; Osram, Berlin, Germany) with an intensity of 13 to 18 W/m^2 (22–33 klux). Cells were synchronized by light/dark changes of 14/10 h and a periodic dilution to a standard cell density of 1×10^6/mL.

Chlorella sorokiniana, strain 211–8k, culture collection Pringsheim (Göttingen, Germany), was cultivated under similar conditions or alternatively at 37 ± 0.5°C and synchronized by light/dark changes of 10/14 h and a periodic dilution to a standard cell density of 1×10^6/mL.

Cell number and cell volume distribution were analyzed using a Coulter Counter (model ZB Industrial) and a Coulter Channelizer C-256 (Coulter Electronic, Luton, Bedfordshire, UK). For statistical treatment (mean cell volume of population, calculated as the median or average of cell volume distribution), data were transferred directly to a microcomputer.

Determination of Concentration-Response Relationships

Algicidal properties of the pyridazinones were evaluated as concentration-response relationships in a 14-h bioassay (t_0–t_{14}) under synchronized conditions using cell volume growth as the integral effect parameter (Altenburger et al., 1990; Faust et al., 1992). Effective concentrations and confidence limits were statistically determined by a two-parameter Logit model (Boedeker et al., 1992; Fahl et al., 1995).

Biotransformation Assay

An appropriate volume of an acetone stock solution of the pyridazinone to be tested was transferred into an Erlenmeyer flask and the acetone was removed by evaporation prior to filling the flask completely with nutrient medium, resulting in a concentration of 50 μM. The flask was then closed and incubated for about 16 h at 45°C in the dark with continuous stirring until the substance was redissolved in the medium. Solutions of P450 inhibitors were prepared in the same way, but they were redissolved with the prepared solution of the pyridazinone. Precise concentrations of the potential inhibitor were adjusted by varying the ratio of these two solutions to provide the solution of substrate/inhibitor used in the inhibitory assay.

Algae were harvested by centrifugation (3250*g*, 5 min), washed twice, resuspended in nutrient medium, and adjusted to a biovolume to medium ratio of 2.0 ± 0.04 μL/mL (average cell volume × cell number). Aliquots of 5 mL of algal suspension were placed in 10-mL centrifuge tubes containing a 15-mm magnetic stirrer bar. Test tubes were placed in a water bath at 28°C with a multipoint magnetic

stirrer (Variomag; H+P, Munich, Germany) adjusted to maximum revolutions per minute and illuminated as described above. It was possible to test up to 48 samples simultaneously. The assay was started by adding 5 mL of substrate/inhibitor solution to each test tube, resulting in (a) a final biovolume to medium ratio of 1.0 ± 0.02 μL/mL, (b) a final concentration of the *N*-dealkylase substrate of 25 μM, and (c) the final concentrations of the inhibitor tested of 0 to 100 μM. After a given period of incubation the algae were pelleted in a precooled (4°C) centrifuge at 3250*g* for 5 min. Supernatants were processed using solid-phase extraction on activated RP-18 cartridges (Merck). Cartridges were washed once with 0.5 mL of double-distilled water and dried with air, and the adsorbed solutes were eluted with 3 mL of acetonitrile. Subsequently, acetonitrile was evaporated by vacuum centrifugation (Speed-Vac; Savant Instruments, Farmingdale, NY) and finally redissolved in 0.2 mL of acetonitrile, followed by HPLC analysis.

HPLC equipment consisted of an L-6200A HPLC pump, an AS-4000 autosampler that included a cooling sample rack, an L-4500 diode array detector, and D-6000 HPLC software (Merck). Separation of the pyridazinones was performed isocratically on a Supersphere RP18 column (125 × 4 mm i.d.) (Merck) with an appropriate guard column (4 × 4 mm i.d.). The composition of the solvent was 40% acetonitrile and 60% H_2O, with a flow rate of 1.5 mL/min. Compounds were detected at 240 nm and quantification was effected by the external standard method. Peak identification was judged by the respective retention times and spectra comparison (220–360 nm).

Protein Separation and Immunoblot Assay

Algae were harvested (3,250*g*, 5 min), washed twice in double-distilled water, and adjusted to a cell number of approximately 1×10^9 cell/mL. For cell disruption, 10 mL of the algae suspension containing 4 mM 4-(2-aminoethyl)-benzolsulfonylfluoride hydrochloride, were passed through a French pressure cell (Aminco, Silver Spring, MD) and subsequently homogenized using a Potter homogenizer. Cell debris were removed by centrifugation (4,000*g*, 5 min). The supernatant was diluted 1:10 with acetone and stored for 15 h. The precipitated proteins were pelleted by centrifugation (10,000*g*, 3 min) and resuspended in SDS sample buffer as described by Fling and Gregerson (1986). All steps were performed at 4°C.

The protein content was determined using the procedure of Neuhoff et al. (1979) and adjusted to 2 mg/mL. Protein separations were performed by SDS-PAGE on 12.5% gels using an electrophoresis cell (Mini Protean II, Bio-Rad). Immediately after electrophoresis the polypeptides were blotted onto PVDF membranes (Immobilon, Millipore, Bedford, MA) using a Mini-Transblot-Transfer cell (Bio-Rad). To avoid artifacts probably caused by highly antigenic sugars, the blots were pretreated with periodate before the incubation with the antibodies (Werck-Reichhart et al., 1993). The polyclonal antisera tested were as follows: the monospecific anti-CA4H (Werck-Reichhart et al., 1993) originally raised against the CA4H (CYP73A1) from *Helianthus tuberosus* and an anti-ECOD (Batard et al., 1995) generated from a 7-ethoxycoumarin-*O*-de-ethylating P450 from *H. tuberosus* (both gifts from the Centre National de la Recherche Scientifique, Strasbourg, France). Also raised against plant P450s were anti-AOS (Lau et al., 1993) generated from a tulip AOS (CYP74) and anti-ARP1 (O'Keefe and Leto, 1989) that were generated from a P450 polypeptide (CYP71A1) from avocado. From two bacterial (*Streptomyces griseolus*) P450 antigens CYP105A1 and CYP105B1, which were characterized as sulfonylurea *N*-dealkylating P450s generated, were the anti-P450 SU1 and anti-P450 SU2, respectively (O'Keefe et al., 1988; Omer et al., 1990). Anti-AOS, anti-ARP-1, anti-SU1, and anti-SU2 were gifts from DuPont. Dilution of each antiserum was 1:2000. Western blot analysis was carried out using swine anti-rabbit alkaline phosphatase conjugate (Dako, Hamburg, Germany).

RESULTS

Algal Toxicity of the Pyridazinone Derivatives

Concentration-response relationships determined for cell volume growth, an integral effect parameter, show increasing toxic properties for both algal species within the congeneric series of pyridazinone herbicides in the order SAN 9774 < metflurazon < norflurazon, the last being the most active compound (Fig. 2). The comparison of the respective EC_{50} values, which are calculated to be 4.3, 0.2, and 0.04 μM in the case of *C. fusca* and 15.8, 4.6, and 0.6 μM for *C. sorokiniana*, gave evidence that *C. fusca* is more susceptible to these agents, particularly to metflurazon. The ratio between the EC_{50}s in the case of metflurazon is about 1:25 (susceptibility of *C. sorokiniana* versus *C. fusca*) and, because of the different slopes of the curves, increases to 1:80 with regard to the respective EC_{10} values.

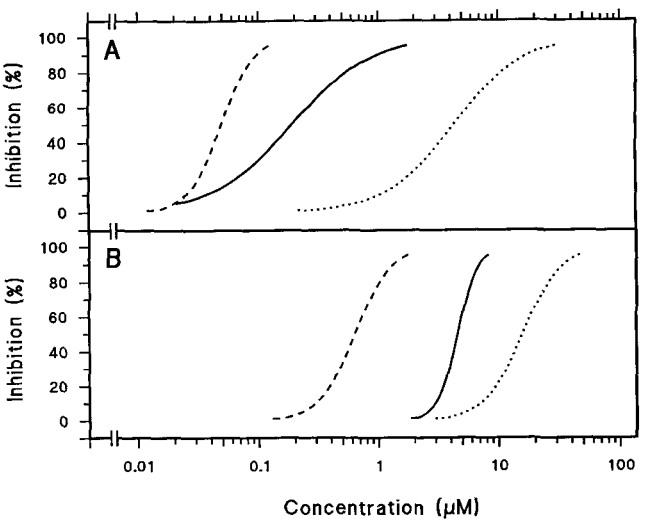

Figure 2. Concentration-response relationships of the pyridazinone herbicides metflurazon (———), norflurazon (– – – –), and SAN 9774 (· · · · · · · ·) for the integral effect parameter cell volume growth of *C. fusca* (A) and *C. sorokiniana* (B) determined after 14 h (t_0–t_{14}) of incubation. Curves were statistically calculated by Logit transformation (Boedeker et al., 1992) of two independent assays, each divided into 14 distinct concentrations at a time.

Biotransformation Studies

With regard to sensitivity, working conditions, and running time, the assay procedure was optimized by using a high biovolume to medium ratio of up to 1.0 μL algae mL^{-1} without additional aeration or CO_2 enrichment. Consequently, cell development was virtually halted.

In many cases, according to the individual mode of action, this approach allows treatment with high concentrations of the agent in question. Both features mean that only 30 min of incubation are required to detect the main biotransformation products in the medium. Typical chromatograms of subsequent HPLC analyses reflect the biotransformation profile of *C. fusca* (the respective profiles of *C. sorokiniana* are quite similar) after treatment with metflurazon or norflurazon (Fig. 3). Using diode array spectrum analysis, we resolved more than six related biotransformation products. After the application of metflurazon, the main product detected is norflurazon, followed by four more hydrophilic compounds (nos. 2–4), including the desmethyl derivative SAN 9774. On the other hand, after treatment with norflurazon at least two additional biotransformation products (nos. 1 and 5) were detected. Compounds 1 and 2 (both very hydrophilic) have been proved to be very unstable when treated with a higher temperature (60°C). They also are very unstable when stored in the organic solvents methanol or acetonitrile. In both cases compounds 1 and 2 are rapidly converted into the desmethyl derivative SAN 9774. The same is true for compound 5, which, when the sample was analyzed immediately after solid-phase extraction, appears to be the major intermediate formed in both algae.

With regard to the biotransformation products 1 and 2, it is important to note that recovery after the solid-phase extraction decreases with decreasing lipophilicity of the analyzed product. Metflurazon and norflurazon are fully retained by the reversed-phase packing of the separation/extraction tubes, resulting in a recovery of 100%. However, recovery of the demethyl derivative SAN 9774 is limited to 80 ± 6%. Thus, the amounts of compounds 1 and 2 detected may reflect only a fraction of the total actually formed.

As shown above, the biotransformation of metflurazon to norflurazon is accompanied by a toxification of both photoautotrophic organisms. Therefore, kinetic aspects of this *N*-demethylation mechanism might be the reason for differences in sensitivity between *C. fusca* and *C. sorokiniana*. Differences in the Michaelis Menten kinetics of the metflurazon-*N*-demethylation (Fig. 4) corroborate such a consideration. K_m(app.) values were calculated to be 1.25 μM for *C. fusca* and 11.1 μM for *C. sorokiniana*. Since these values were estimated from an in vivo assay, they integrate all kinetic processes involved, including substrate absorption, enzymatic transformation, and release of the product.

Synchronized cultures were used to determine characteristic features in *N*-demethylase activity along the pro-

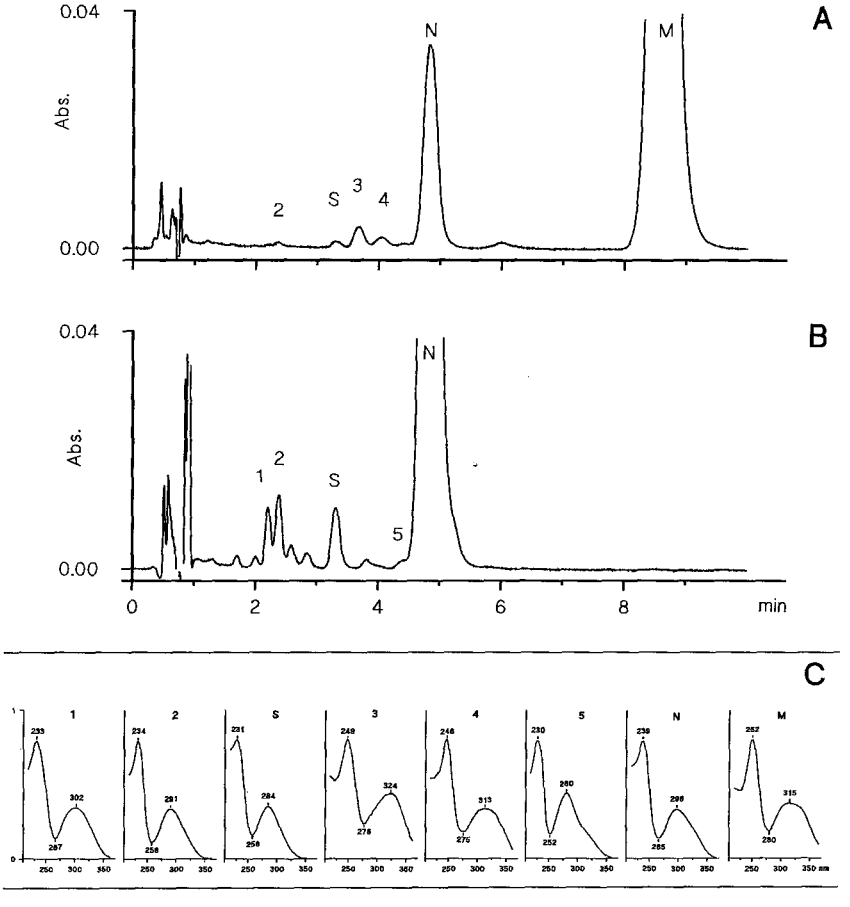

Figure 3. Chromatograms of HPLC analyses of typical biotransformation assays with *C. fusca* after the application of metflurazon (A) and norflurazon (B), each at 25 μM and an incubation period of 2 h. The biotransformation products formed are characterized by the respective normalized UV spectra (C). M, Metflurazon; N, norflurazon; S, SAN 9774. Abs., Absorbance.

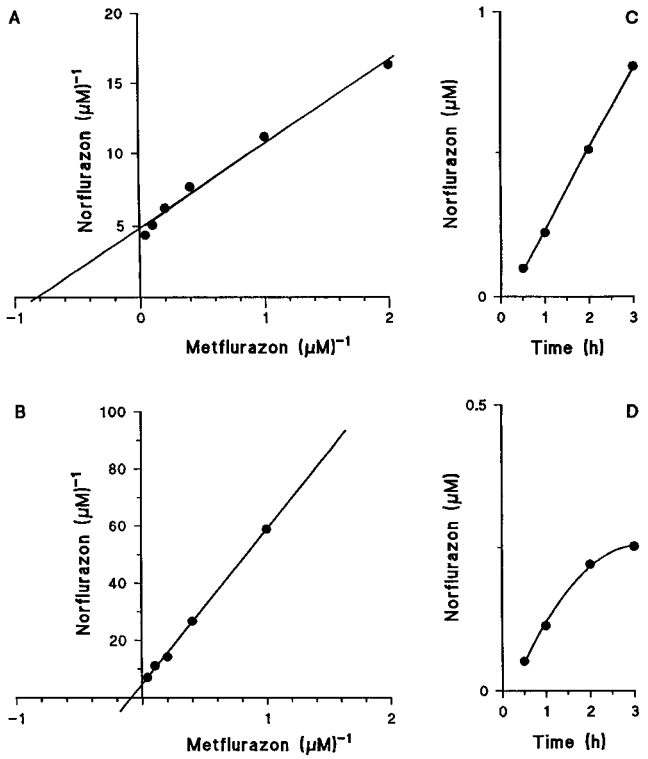

Figure 4. Lineweaver-Burk plots of the apparent Michaelis Menten kinetics of the in vivo *N*-demethylation of metflurazon in *C. fusca* (A) and *C. sorokiniana* (B). Algae were harvested at t_4 of the cell cycle and adjusted to a biovolume to medium ratio of 1.0 μL/mL. C and D, Linearity of the respective biotransformation process versus time and a metflurazon concentration of 25 μM.

cess of cell maturation (Fig. 5). Under synchronized conditions and a cultivation temperature of 28°C, the cell cycle of both algae is characterized by an exponential growth phase for 14 h in light, followed by the proliferation of new autospores in the dark. In the case of *C. sorokiniana*, the autospore proliferation takes place in the first 2 to 3 h after the light to dark change (t_{14}–t_{17}), whereas *C. fusca* proliferates autospores later, between t_{21} and t_{24}. During the exponential growth phase, the metflurazon-*N*-demethylase activity of *C. fusca* increased supraproportionately. The highest turnover is reached at t_{10} of the cycle; in the following 6 h the activity decreased to the original value. In contrast, the enzymatic activity of *C. sorokiniana* rapidly increased in the first hours of the growth phase and there was a distinctive peak of activity at approximately t_8 of the cycle, followed by a decrease just as rapid as the former increase. Subsequently, before the original value was reached, a second peak of metflurazon-*N*-demethylase activity was observed at t_{14}, which, however, was less distinct. Throughout the growth period, except for the 3 h of maximum activity of *C. sorokiniana*, the rate of metflurazon-*N*-demethylation of *C. fusca* was higher by about 2-fold.

Effects of Cyt P450 Inhibitors and Alternative Substrates

The rate of algal metflurazon demethylation was measured in the presence of ABT, BAS 111..W, tetcyclacis, and PBO (Table I), all known inhibitors of the P450-mediated monooxygenase systems. Each of these compounds reduced metflurazon *N*-demethylation, as indicated by diminished quantities of norflurazon that were formed. The metflurazon demethylation in *C. sorokiniana* was generally more susceptible to these agents. The biotransformation of norflurazon was also reduced in the presence of P450 inhibitors (data not shown). However, according to the more complex biotransformation profile, this effect was clearly discernible only by using the disappearance of norflurazon itself as the reference point.

The rate of metflurazon demethylation was also determined in the presence of potentially competitive substrates, the natural substrate cinnamic acid and the xenobiotic 7-ER. Cinnamic acid decreased the demethylation in both algae, but 7-ER increased the activity. The amount of stimulation depended on the concentration of 7-ER (Fig. 6); 5.0 μM increased the formation of norflurazon by *C. fusca* 2-fold and by *C. sorokiniana* more than 10-fold. The increased formation of norflurazon was accompanied by a more rapid disappearance of the substrate metflurazon out of the medium. *C. sorokiniana* (1.0 μL/mL) treated with 5.0 μM 7-ER removed 50% of the simultaneously applied metflurazon within 1 h. The stimulating effect itself is induced immediately after treatment. With 0.5 μM 7-ER, the stimulating effect lasts more than 2 h following a second-order regression (Fig. 7). The degradation of norflurazon was also stimulated (data not shown). In this case, the major

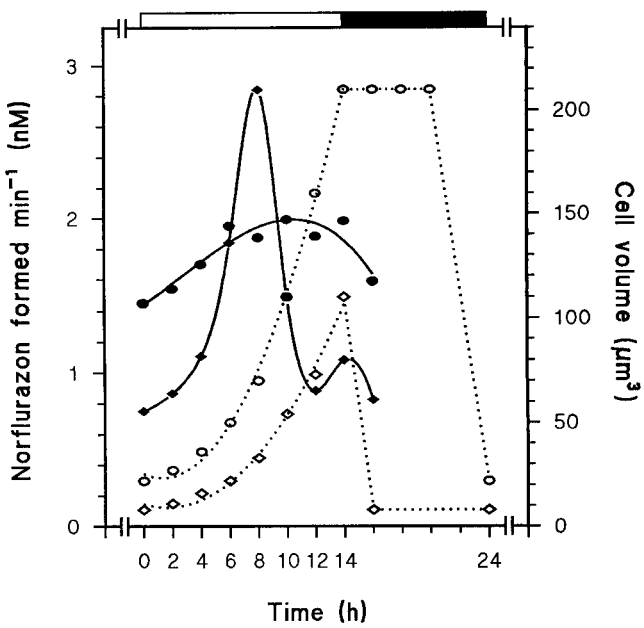

Figure 5. Formation of norflurazon (black symbols) from metflurazon (4.0 μM) within the cell cycle of *C. fusca* (circles) and *C. sorokiniana* (diamonds). White symbols represent the median of the cell volume distribution at t_x of the cell cycle. Bars at the top indicate the period of illumination (white bar) and darkness (black bar) under synchronized conditions. Algae were harvested at t_x of the cell cycle and adjusted to a biovolume to medium ratio of 1.0 μL/mL. The duration of assays was 1 h. Conditions were as stated in the text. Values are means of two samples.

Table I. *Light dependence and effect of various inhibitors and possible substrates of plant Cyt P450s on the algal* in vivo *N-demethylation of metflurazon (25 μM)*

Durations of the biotransformation assays were 60 min. Inhibitors and metflurazon were applied simultaneously. Assay conditions were as stated in the text. Values are means of two samples.

Inhibitor/Substrate	Concentration	Relative Activity	
		C. fusca	C. sorokiniana
	μM	%	
H_2O			
Light		100	100
Dark		20	53
ABT	20	85	57
	50	70	30
	100	28	6
BAS..111W	20	90	64
	50	71	35
	100	55	27
Tetcyclacis	20	80	73
	50	72	45
	100	53	40
PBO	20	79	54
	50	70	28
Cinnamic acid[a]	10	100	95
	50	35	40
	100	10	30

[a] According to its pK_a of 4.6, assays using cinnamic acid were performed at pH 5.0, which is tolerated by both algal strains.

intermediate formed was the structurally nonidentified compound 5, which undergoes rapid conversion to SAN 9774.

With respect to the increase in cell volume of *C. sorokiniana*, the algicidal properties of BAS..111W, ABT, cinnamic acid (nonobservable effect concentrations, 0.1 mM), and 7-ER (nonobservable effect concentration, 1.0 μM) enabled the study of potential coergetic effects on algal growth inhibition caused by metflurazon and norflurazon. In this regard we compared the effects of appropriate binary mixtures of pyridazinone/P450 effectors with those caused by the pyridazinone derivative alone (Fig. 8). The toxic behavior of metflurazon was sharply reduced in the presence of the P450 inhibitors BAS 111..W and ABT, as well as in the presence of cinnamic acid. On the other hand, inhibition affected by norflurazon was reduced by a simultaneous application of 7-ER, thus validating its stimulatory properties on norflurazon degradation, which is noted above.

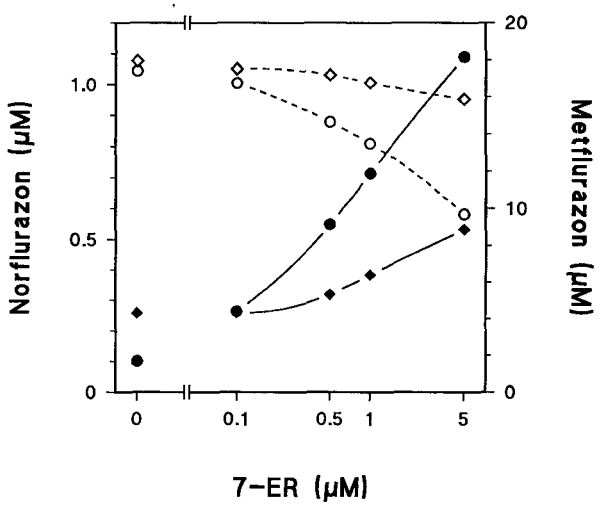

Figure 6. Effect of 7-ER on the formation of norflurazon by metflurazon-*N*-demethylase in *C. fusca* (◆) and *C. sorokiniana* (●) and the remaining metflurazon (◇, ○). The biovolume to medium ratio was adjusted to 1.0 μL/mL. The duration of assays was 1 h. Values are means of three samples. Conditions were as stated in the text.

Antigenic Cross-Reactivity of Algal Proteins with Heterologous Polyclonal Antibodies

It is not possible to demonstrate the presence of Cyt P450s in algal preparations by CO-difference spectra because of interfering pigments. Thus, we attempted to detect P450s in crude preparations of algal proteins using antibodies raised against plant and bacterial isoforms of P450. Proteins were separated by SDS-PAGE and blotted prior to the immunoassay. Cross-reactivity with algal proteins in the range of 30 to 70 kD was found for all antisera tested; however, the immunoblots indicate differences between both algal strains (Fig. 9).

Some similarities in the cross-reactivity patterns (47, 48.5, 54.5, 63, and 66 kD) of polyclonal antibodies originally raised against P450s from plants (anti-CA4H, anti-ECOD, anti-ARP1, and anti-AOS, respectively) were detected for *C. fusca*, however, the intensities differed. The 47-kD polypeptide was strongly recognized by anti-AOS, whereas cross-reaction of all other antibodies at 47 and 48.5 kD were weak. The 54.5-kD protein showed the strongest cross-reactivity for anti-CA4H and anti-ECOD. Contrasting cross-reactivities were given for polypeptides of 63 and 66 kD, which were both recognized by anti-CA4H and anti-ARP1; anti-AOS reacted only at 63 kD and anti-ECOD

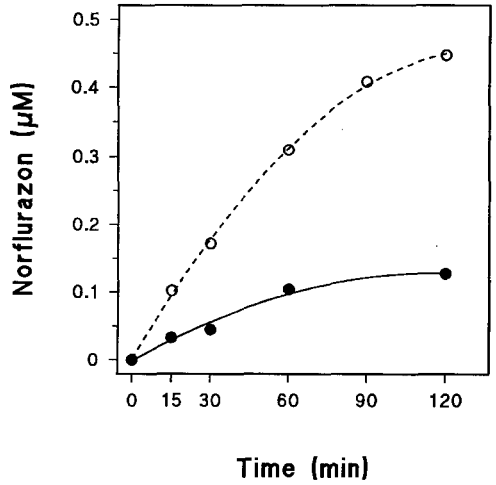

Figure 7. Time course of the stimulation of metflurazon-*N*-demethylation in *C. sorokiniana* induced by 7-ER. The biovolume to medium ratio was 1.0 μL/mL. Conditions were as stated in the text. ●, 20 μM metflurazon; ○, 20 μM metflurazon plus 0.5 μM 7-ER.

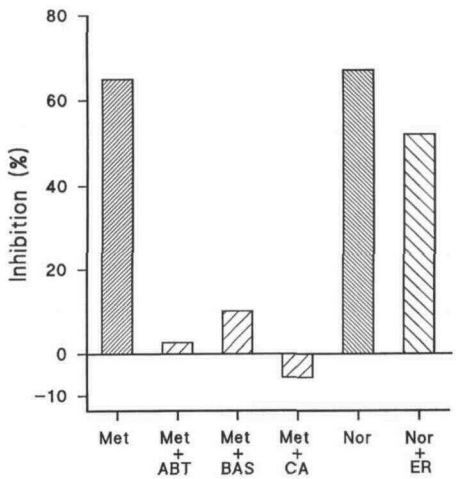

Figure 8. Antagonistic effects of Cyt P450 interacting agents on the inhibition of cell volume growth of *C. sorokiniana* caused by metflurazon (Met; 5.3 μM) or norflurazon (Nor; 1.5 μM). Data were determined in a 14-h bioassay (t_0–t_{14}). Conditions were as stated in the text. ABT, BAS, and cinnamic acid (CA) were each at 0.1 mM. 7-ER (ER) was at 1.0 μM.

showed no affinity to algal proteins in this size range. Cross-reactivity in the size range less than 46 kD was detected for anti-CA4H at 45 kD, anti-ECOD at 36 kD, and anti-AOS very strongly at 40 kD.

In the corresponding blots of *C. sorokiniana*, anti-CA4H reacted with only a 63- and 66-kD protein. Anti-ARP1 also showed weak cross-reactivity at 47 and 48.5 kD. In contrast to these results, anti-AOS produced a strong cross-reaction with a 50.5-kD protein and a weak reaction with proteins at 47 and 52 kD. Weak cross-reactivity was observable against anti-ECOD only at 48 and 50.5 kD.

Antisera originally raised against two herbicide-biotransforming P450s from *Streptomyces griseolus* (SU1 and SU2) also gave distinct patterns of cross-reactivity with algal proteins. The SU2 type reacted strongly with a 52-kD protein of *C. sorokiniana*, but no cross-reactivity was detected with *C. fusca*. The SU1 type, however, showed affinity in both algae to a polypeptide of 65 kD and in *C. fusca* also at 54, at 40, and very strong at 37 kD.

DISCUSSION

Concentration-response data of kinetic properties of *C. fusca* and *C. sorokiniana* indicate that the relatively more efficient *N*-demethylation of metflurazon by *C. fusca* contributes to the higher sensitivity to this compound determined in this organism. The biotransformation product formed, norflurazon, is the more biologically active compound in both algae. This point was confirmed by quantitative structure activity relationship analysis. Biochemical inhibition of the *N*-demethylation step diminished toxicity to both algae, in the case of *C. sorokiniana* almost completely, indicating that metflurazon itself is not or is only slightly toxic. The distinct susceptibility of *C. fusca* to this pro-herbicide is, in addition, caused by an even higher sensitivity to the biotransformation product norflurazon. The wide concentration range of activity of metflurazon determined for *C. fusca* and described by the low slope of the respective concentration-response curve is attributed to the comparative low K_m(app.) value determined for the metflurazon-*N*-demethylase of this alga. Norflurazon itself is biotransformed to further intermediates, including a subsequent *N*-demethylation step toward the less toxic SAN 9774.

Five more biotransformation products have been detected but not yet structurally identified. However, with regard to compounds 1 and 2 (Fig. 3), their rapid conversion to SAN 9774, spectral properties, and retention behavior in reversed-phase HPLC might indicate the formation of phase-II conjugates of SAN 9774. Plant constituents used for phase-II conjugation of xenobiotics are sugars, amino acids, malonic acid, or the reduced tripeptide glutathione. Aside from conjugates of glutathione, an unusual constituent for conjugation of P450 phase-I intermediates, conjugations are documented to be in parts reversible (Cole, 1994). The spectral shift to a shorter wavelength, which has been observed and is caused by successive demethylation of the 5-dimethylamino group, has been confirmed by molecular orbital calculations (D. Schlettwein, personal communication). A subsequent conjugation at position 5′ even with a nonchromophoric substituent should be accompanied by a bathochromic shift. This was observed for compounds 1 and 2 in relation to the spectrum of SAN 9774 (Fig. 3C). Conjugation with each of the plant constituents in question should at least be accompanied by a markedly increased hydrophilicity. Similar considerations might be appropriate for compounds 3 and 4 in relation to norflurazon.

An involvement of the P450 monooxygenases for both algal strains is indicated by the inhibitory effect on metflurazon-*N*-demethylation determined for all of the P450 in-

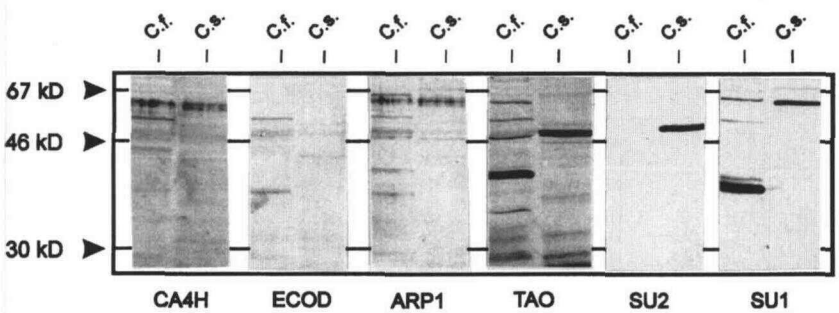

Figure 9. Western blot analysis of protein preparations of *C. fusca* (C.f.) and *C. sorokiniana* (C.s.) with antisera raised against Cyt P450 isoenzymes from plants (CA4H, ECOD, ARP1, and AOS) and *S. griseolus* (SU1 and SU2). The dilution of each antiserum was 1:2000. Forty micrograms of protein was loaded in the lanes indicated.

hibitors used, including the mechanism-based types ABT and PBO. Furthermore, light dependence of the in vivo *N*-demethylase activity assay (Table I) suggests insufficient reducing power in the dark caused by a limited regeneration of cytosolic NADPH + H^+, the essential co-substrate of P450 monooxygenases. With regard to norflurazon-*N*-demethylation, an involvement of P450 appears likely. However, it is not judged clearly because there are further biotransformation products and their association to SAN 9774 is not entirely clear.

Another point concerns the stimulation of algal *N*-demethylation caused by 7-ER, a xenobiotic model substrate for the determination of P450-mediated phase-I biotransformation activity. The results are comparable to those of a previous study, in which algal ECOD was also shown to be more than 10 times faster in the presence of 7-ER (Thies and Grimme, 1995). The time course of metflurazon-*N*-demethylation demonstrated that the stimulation starts immediately after the application of 7-ER (Fig. 7), which excludes an induction mechanism associated with increased protein expression. Therefore, we suggest that this stimulating effect might be caused by a redox-triggered activation. A similar turnover-stimulating effect was demonstrated for cumene hydroperoxide, which increased the *N*-demethylation of *p*-chloro-*N*-methylaniline, catalyzed by an avocado P450 (associated with the ARP-1 antisera, see below), by a factor of 10 (O'Keefe and Leto, 1989). In this case, the enhancement was attributed to an alternative peroxygenative activity of the P450, which implies that no requirement exists for reduced pyridine nucleotides, for molecular oxygen, or for a reductase system. However, 7-ER contains no peroxide moiety that could serve as an electron donor; therefore, alternative explanations, e.g. due to a partial permeabilization of the algal membrane, may be possible as well.

It must be noted that in the case of eukaryotic photoautotrophic algae, which represent the main group of primary producers in aquatic systems, references to P450 are hitherto rare (Ladouceur et al., 1990; Thies and Grimme, 1995) and systematic research is lacking. Further discussion (below) is related to properties reported for plant P450s, because of the position of those algae in the phylogenetic tree between prokaryotic photoautotrophic cyanobacteria and higher plants.

The characteristic cyclic behavior of *N*-demethylation activity exhibited during the cell cycle indicates the presence of regulatory mechanisms associated with enzyme expression and activity. A comparison with increase in cell volume during the growth phase (t_0–t_{14}) discloses a supraproportional increase of metflurazon-*N*-demethylase activities within the first 8 h, which might reflect increased expression of the corresponding P450 enzymes. This period was followed by a subsequent decrease in activity, even when cell volume was still increasing exponentially. This distinct variation indicates that the rate of metflurazon *N*-demethylation is more than just a function of cell volume. Recently, we reported (a) similar relationships concerning the variation of algal alkoxycoumarin- and alkoxyresorufin-*O*-dealkylases (Thies and Grimme, 1995) and (b) concerning the process of cell division, an increased susceptibility to P450 inhibitors, especially in the last hours of the exponential growth period (Thies and Grimme, 1996). This point suggested that, during this particular period of the cell cycle, competing P450-mediated metabolic reaction(s) take place. When adapted to the present investigation, these observations imply that *N*-demethylation of metflurazon might be catalyzed by P450 enzyme(s) usually involved in the metabolism of physiological substrate(s). Such a double function of plant P450s is well documented for a lauric acid hydroxylase from wheat, which hydroxylates the herbicide diclofop as well (Zimmerlin and Durst, 1992), and for the avocado P450 CYP71, normally associated with fruit ripening, which mediates *N*-demethylation of *p*-chloro-*N*-methylaniline (Bozac et al., 1992). Thus, it is likely that those P450s of algae involved in physiological processes can also participate in the biotransformation of foreign compounds. According to an implied high substrate specificity (Durst and Benveniste, 1993) of plant P450s, the participation in xenobiotic biotransformation should therefore be restricted to analog-induced or accidental reactions. The reducing effect on metflurazon-*N*-demethylation demonstrated here for cinnamic acid also confirms such a consideration for unicellular green algae. Cinnamic acid is the physiological substrate of a plant P450, CA4H (CYP73A1), which has been reported to be highly specific for this natural compound. Nevertheless, the same enzyme was also shown to oxygenate several xenobiotics, including *O*-dealkylation of 7-ethoxycoumarin and *N*-demethylation of *p*-chloro-*N*-methylanilin (Pierrel et al., 1994).

Many suitable techniques used in P450 research, e.g. CO-difference spectroscopy, spectral substrate-binding studies, or immunoinhibition assay, depend on the availability of microsomal fractions. Furthermore, spectral photometric approaches require achromatic microsomes, which restrict the origin of material, in the case of plants, to bleached or etiolated tissues. In the case of the unicellular green algae used in our study, microsome preparation is prohibited because *Chlorella* spp. resists virtually all methods of cell disruption and homogenization. The only (partially) successful method is the French press, but the volume of an algal suspension required to be equivalent to 10 g of plant tissue would still necessitate a volume of 200 mL. In addition, algal microsomal preparations will be contaminated with pigments, which interfere in spectral photometric assays.

Thus, proof of antigenic cross-reactivity of immobilized algal proteins with antibodies raised against different P450s was chosen as a suitable way to obtain evidence of P450 in *Chlorella* spp. However, interspecies cross-reactivity between denatured plant P450s has been reported to be very limited. Higashi et al. (1985), using a polyclonal antibody raised against the major P450 in tulip bulbs, recently characterized as an AOS (Lau et al., 1993), observed no cross-reactivity, even with closely related species. Limited cross-recognition on western blots subsequent to SDS-PAGE also has been described for antibodies raised against CA4H from *H. tuberosus* (Werck-Reichhart et al., 1993). Nevertheless, all antisera tested in this study and originally

raised against plant P450s, including anti-AOS and anti-CA4H, showed cross-recognition of several immobilized algal proteins within the M_r range of 46,000 to 67,000. Typical molecular masses of isolated plant P450s are reported in the size range of 46 to 56 kD (Bolwell et al., 1994).

However, with regard to the dilution of the primary antibodies, which was 1:2000 in all cases, the specificity of the cross-reactivity might be in question. Dilutions cited for western blotting in the original publications ranged between 1:1,000 (anti-ARP-1) and 1:10,000 (anti-CA4H); therefore, nonspecific binding cannot definitively be excluded.

Immunoreactions of anti-SU1 and anti-SU2 with algal polypeptides reveal strong cross-recognition of eukaryotic algal proteins. Anti-SU1 and anti-SU2 are cross-reactive to the corresponding P450 antigens from the prokaryote *S. griseolus* and have never demonstrated cross-reactivity to any plant protein (D.P. O'Keefe, personal communication) with the exception of anti-SU2, which was recently reported to recognize a tobacco benzoate hydroxylase (León et al., 1995).

In summary, the results of the immunoanalyses suggest the presence of diverse P450s in both algal species; beyond this, the distinct patterns of cross-reactivity indicate interspecies differences even between *Chlorella* ssp. However, with regard to algal xenobiotic biotransformation, it is not possible, based on the present point of view, to attribute metflurazon-*N*-demethylation to particular protein(s). It appears likely that the extensive antigenic cross-reactivity (supposedly more limited at first) is caused by more distinct epitope homologies between the P450s of algae and higher plants as well as algae and bacterial P450s. However, to support this consideration, the extent of domain homology or conservation needs to be validated by enzyme purification and estimation of sequence similarities. In any case, algae appear to be appropriate organisms for further approaches toward the study of P450s, including function, structure, regulation, and evolution.

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